POTENTIAL MECHANISM FOR DIFFERENTIATING CONSPECIFIC AND HETEROSPECIFIC OFFSPRING OF COMPETING BLOW FLIES (DIPTERA: CALLIPHORIDAE)

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Potential Mechanism for Differentiating Conspecific and Heterospecific Offspring of Competing Blow Flies (Diptera:Calliphoridae). (May 2015)

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Regulatory mechanisms related to blow fly (Diptera: Calliphoridae) attraction to ephemeral resources provide a model for understanding nutrient cycling, food web dynamics, and decomposition ecology. The initial colonization of an ephemeral resource by blow flies often results in competition between heterospecifics and conspecifics. *Cochliomyia macellaria* (Fabricius, 1775) and *Chrysomya rufifacies* (Macquart, 1842) are two necrophagous blow fly species that compete for carrion resources in Texas. While visual and olfactory cues play a role in competition between each blow fly species, the metabolic roles of microbial communities present on *C. macellaria* and *C. rufifacies* may aid in the attraction and colonization of a resource. The Biolog Ecoplate™ is a phenotypic microarray that can be used to assess the microbial metabolic community profiles (MMCPs) of an environment or organism of interest. This study used Biolog Ecoplates™ to analyze similarities and dissimilarities of the bacterial metabolic community profiles present on the eggs, 1st instars, and 2nd instars of *C. macellaria* and *C. rufifacies*. Overall, MMCPs differed in heterospecifics at the first instar stage, signifying that bacterial communities began to diverge once the egg stage was complete. Additionally, bacterial communities of conspecifics not only diverged between each immature stage but showed divergence from generation to generation, signifying that the more exposure to the

environment an organism had, the less homogeneous a population became. The differences in the MMCPs of heterospecific and conspecific immature stages signal a potential mechanism of bacterial community shifts in order to mediate competition and co-existence between these competing species.

DEDICATION

I dedicate this research to my parents, Randy and Vee Thornton. This research would have not been a success if it were not for their endless positivity, patience, and loving support. I also dedicate this research to Shelley Meyers for teaching me perseverance and lighting the flame that is my passion for science.

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NOMENCLATURE

MMCP	Microbial Metabolic Community Profile
EFP	Ephemeral Resource Patch
L:D	Light: Dark Cycle
NMDS	Nonmetric Multidimensional Scaling
PERMANOVA	Permutational Multivariate Analysis of Variance
ISA	Index Species Analysis
mPMI	Minimum Post-Mortem Interval

CHAPTER I

INTRODUCTION

Ephemeral resources

Ephemeral resources experience rapid decomposition rates and are a host to a wide diversity of microorganisms and macroinvertebrates (Finn 2001). Fruits, manure, leaf piles, crops used in agricultural and carrion are ephemeral resource patches (EFPs) that contribute to the fluctuations of species richness and diversity of various ecosystems while also providing temporary sustenance, refuge, and breeding grounds for passing organisms (Jonsson 2005). Beaver (1977) described these EFPs as small 'islands' within a community that are neither self-sustaining nor long lived, requiring rapid dispersal into the surrounding environment after the depletion of the EFP. The influxes of these unpredictable EFPs create intense conspecific and heterospecific competition for available resources which influences individual fitness (Krause and Ruxton 2002).

Blow flies (Diptera: Calliphoridae)

There is a distinct faunal succession associated with carrion that encompasses a wide variety of arthropods, many of which are necrophagous, sarcosaprophagous or predatory in nature (Greenburg 1991). Blow flies, in particular, are necrophilous insects that are frequently the first to colonize carrion, and therefore are of particular interest when studying decomposition ecology (Amendt et al. 2004). Two blow flies commonly found in Texas, *Chrysomya rufifacies* (Macquart, 1842) (Diptera: Calliphoridae) and *Cochliomyia macellaria* (Fabricius, 1775) (Diptera: Calliphoridae), are of interest when studying conspecific and heterospecific effects of

species competing for resources. Both *C. rufifacies* and *C. macellaria* are found in close association with carrion but vary temporally in adult arrival and oviposition (Brundage 2014, Eberhardt and Elliot, 2008). *C. macellaria* has been described as acting as a primary colonizer (Flores 2010), while *C. rufifacies* acts as a secondary colonizer (Fuller 1934). The unique temporal activities of each blow fly species favors the predaceous larvae of *C. rufifacies* that feed on the larvae of *C. macellaria* (Baumgartner 1993). The predatory interaction of *C. rufifacies* on *C. macellaria* greatly affects the fitness of each species when in the presence of the other (Brundage et al. 2014).

Carrion detection, location, and discrimination mechanisms

Immediately upon the death of an organism, *C. rufifacies* and *C. macellaria* undergo a detection phase of carrion that encompasses two stages: activation and searching. When visual or olfaction cues have been triggered, blow flies can utilize these stimuli to locate new nutrient patches (Tomberlin et al. 2011a).

Visual

The compound eye, made up of ommatidia, is a vital organ in blow flies that allows for visual detection of resource patches (Sukontason et al. 2008). Visual cues have been described as playing an important role in the selection of a landing site on carrion and have been characterized as being closely linked with olfaction in detection at close range (Wall and Fisher 2001).

Olfaction

C. rufifacies and *C. macellaria* locate carrion by utilizing olfactory cues triggered by volatiles emitted from decaying materials (Wall and Fisher 2001) as well as volatiles produced by conspecific and heterospecific female oviposition on the resource (Brundage 2012, Rosati and

Laerhoven 2007). Many volatiles are produced by bacteria associated with the various fly stages and are used by adult flies to assess the quality of a resource for their offspring (Asher and Wall 1994). Ma et al. (2012) determined that the quorum sensing activity of bacteria acts as interkingdom communication between bacteria and dipteran species. Mohr and Tomberlin (2014) also suggested that many of the volatile shifts present on carrion could potentially be explained by microbial community shifts on either the carrion itself or the immature stages of conspecific and heterospecific blow flies.

Avoiding competition by shifting microbial community

The ubiquity of microbial communities gives rise to a diverse array of functional roles in any given ecosystem (Ellis-Evan et al. 1998; Brock 1978). For instance, microorganisms are an important trophic level in decomposition ecology, often mediating the decomposition rates of carrion (Tomberlin et al. 2011b; Pechal et al. 2013). The close association of arthropods and microbes to carrion leads to various interkingdom interactions such as the production of bacterial toxins to deter eukaryotic organisms from competing for a resource (Janzen 1977). Recent studies have shown carrion microbial communities influence the physiology and behavior of necrophagous insects through mechanisms of eavesdropping on microbial community communication (Ma et al. 2012; Tomberlin et al. 2012). Pechal et al. (2013) demonstrated that microbial succession on carrion may influence the arrival of competing blow fly species. If microbial succession on carrion mediates competition between blow fly species, calliphorids may shift their bacterial communities to affect the arrival of competing blow flies to a resource. Singh et al. (2014) demonstrated that structural characteristics of bacterial communities can be used to differentiate two competing blow flies, *Lucilia sericata* (Meigen, 1826) (Diptera:

Calliphoridae) and *Lucilia curpina* (Wiedemann, 1830) (Diptera: Calliphoridae); therefore another approach to differentiating competing blow fly species could lie within the metabolic or physiological profiling of microbial communities. The function of microbial communities can be assessed using Biolog EcoPlates™ (Weber and Legge 2010). These microarrays provide microbial metabolic profiles (MMCPs) that are determined by the differential use of environmental carbon sources (Pechal et al. 2013; Insam and Goberna 2004). The aim of this study was to use MMCPs to characterize and differentiate various conspecific and heterospecific immature stages of *C. macellaria* and *C. rufifacies* in order to gain insight into the functional roles bacteria play in mediating arrival to an ephemeral resource in competing blow flies.

CHAPTER II

MATERIALS AND METHODS

Adult colony maintenance and sampling

C. macellaria and *C. rufifacies* larvae were collected from swine carrion in Brazos County, Texas, USA during the summer of 2014 and were reared in a growth chamber at approximately 25^{o}C- 27^{o}C with a 12:12 h L:D interval at approximately 60-70% RH. Each colony was contained in separate, 30 x 30 x 30 cm mesh cages (Bugdorm™, Taiwan). Adult flies were fed a mixture of water, honey, and granulated sugar *ad libitum*; bovine blood was also provided similarly in a Petri dish (Boatright 2009; Ma et al. 2012). Each fly population was given approximately 30 g liver in a 90 ml Dixie cup as an oviposition substrate. The liver was monitored every 6 h for oviposition for a 24 h period. Approximately 0.05 g eggs were collected aseptically (sterile applicator with cotton bud) for Biolog assessment while remaining eggs were left and monitored every 5 h until hatch occurred. This process was repeated for a second generation per fly species with the addition of the collection of 0.05 g of second instars in the second generation.

Biolog assessment

The 0.05 g eggs collected for the Biolog assessment were placed in a Falcon 50 ml conical centrifuge tube containing 40 ml of sterilized 25% ringer solution and 10 sterilized glass beads. The eggs were homogenized for 2 min using a vortex. The homogenized sample was then centrifuged at 1000 g for 2 min. The supernatant was poured into a sterile Petri dish and 100 µl aliquots were added to each well of a Biolog Ecoplate™. This procedure was repeated three

times for each generation of each fly species. The plates (three per species) were incubated at 25^{o}C and read using a Sunrise^{TM} spectrophotometer (Tecan Group Ltd., Mannedorf, Switzerland) at 12 h intervals for 10 readings, or until each plate reached 0.7 optical density (Stefanowicz 2006). First and 2nd instars for each species were examined using the methods previously described for eggs. The liver that was used to collect eggs was used as a control. Approximately 1.0 g (n=3) was retrieved from a liver sample prior to use for collecting eggs and was processed using the methods described above.

Data Analysis

All Biolog data were analyzed using the statistical program R to assess dissimilarities between the microbiomes of *C. macellaria* and *C. rufifacies* (Pechal et al. 2013, R Development Core Team 2010). Bray-Curtis distance with nonmetric multidimensional scaling (NMDS) and 999 permutations using the Vegan library in R was used to visualize microbial metabolic community profiles (MMCP) across developmental stages and species (R Development Core Team 2010). PERMANOVA is a nonparametric technique that differentiates groups using a dissimilarity matrix (McCune and Grace 2002, Pechal et al. 2013). The adonis function in R performed this analysis using the vegan library and determined significant microbial functional differences between the two fly species. Indicator Species Analysis (ISA) was used to identify the most utilized carbon source of the eggs and first instars of *C. macellaria* and *C. rufifacies*.

CHAPTER III

RESULTS

Heterospecific microbial metabolic community profiles

Across generations

C. macellaria and *C. rufifacies* microbial metabolic community profile (MMCP) similarity distances are presented in Figure 1. Across two generations MMCPs are significantly (df = 6, F = 1.87, P = 0.013) different between *C. macellaria*, *C. rufifacies* (i.e. eggs and first instars), and the liver control. An index species analysis (ISA) showed that D-Xylose, D-L-Glycerol Phosphate, Glucose Phosphate, and Pyruvic Acid Methyl Ester were significant (P > 0.001) indicators of MMCP dissimilarities for *C. macellaria*, *C. rufifacies*, and the liver control. The MMCPs of the first instars of *C. macellaria* and *C. rufifacies* were the only significant (df = 1, F = 1.99, P = 0.019) heterospecific life stage. N-Acteyl-D-Glucosamine was the indicator for MMCP dissimilarities of *C. macellaria* and *C. rufifacies* first instars. The MMCPs for heterospecific eggs of *C. macellaria* and *C. rufifacies* were not significant (P > 0.05) across generations.

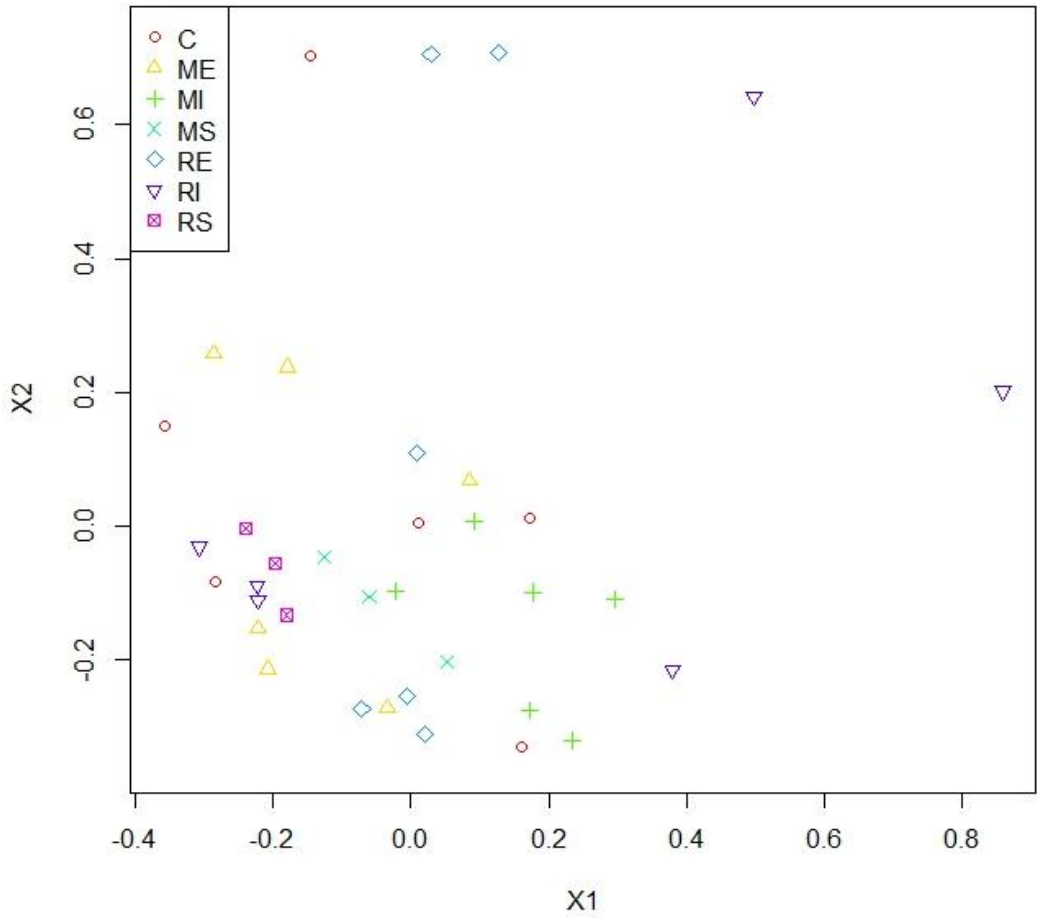

Figure 1. A PERMANOVA scatterplot of treatments across all generations (C = control, ME = *C. macellaria* egg, MI = *C. macellaria* first instar, MS = *C. macellaria* second instar, RE = *C. rufifacies* egg, RI = *C. rufifacies* first instar, RS = *C. rufifacies* second instar).

Generation one

There were no significant ($P > 0.05$) differences between the MMCPs of *C. macellaria*, *C. rufifacies* (i.e. eggs and first instars), and the liver control. The MMCPs for heterospecific first instars were the only significant (df = 1, F = 1.99, $P = 0.019$) life stage in generation one. Figure 2 presents the MMCP similarity distances for generation one.

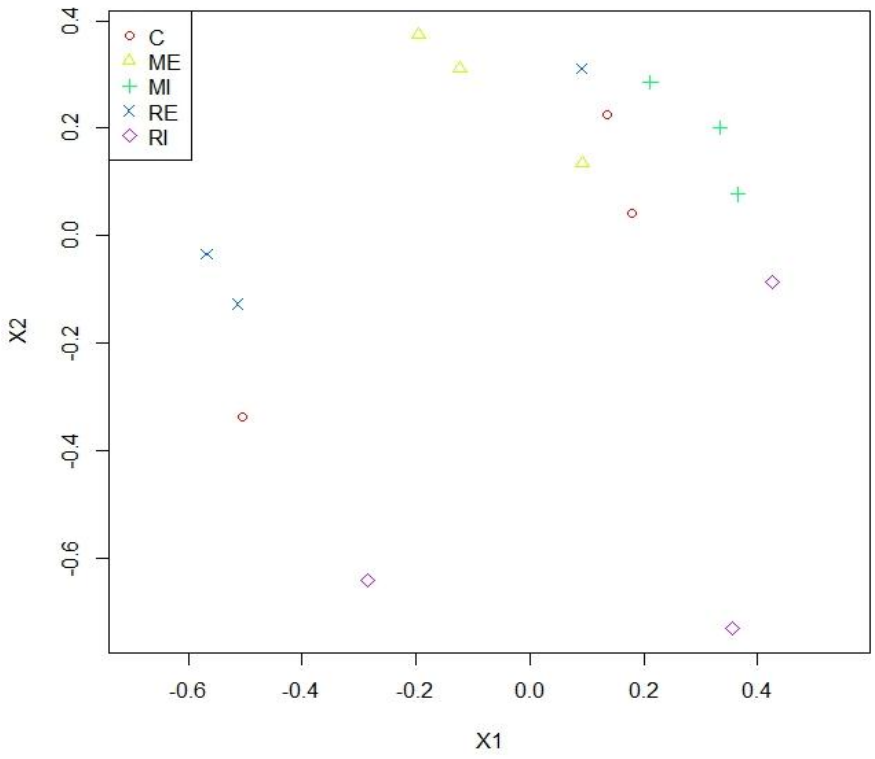

Figure 2. A PERMANOVA scatterplot of generation two treatments (C = control, ME = *C. macellaria* egg, MI = *C. macellaria* first instar, RE = *C. rufifacies* egg, RI = *C. rufifacies* first instar).

Generation two

The overall MMCPs of *C. macellaria*, *C. rufifacies* (i.e., eggs, first and second instars), and the liver control were significantly (df = 6, F = 3.15, P = 0.004) different in generation two. The MMCPs for heterospecific eggs were significant (df = 1, F = 4.79, P = 0.001) with an ISA presenting no carbon indicators of egg MMCPs. The MMCPs for heterospecific first instars were also significantly (df = 1, F = 18.26, P = 0.001) different with an ISA presenting no carbon indicators of first instar MMCPs. The MMCPs of heterospecific second instars were not significantly (P > 0.05) different. Figure 3 presents the MMCP similarity distances for generation two.

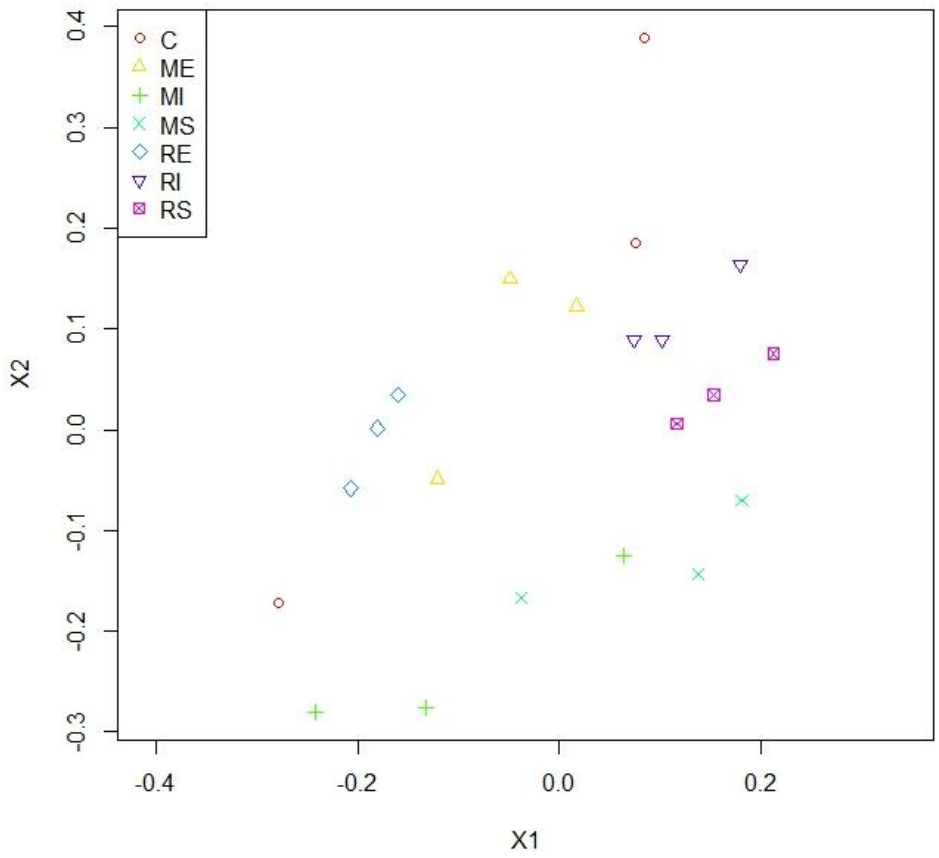

Figure 3. A PERMANOVA scatterplot of generation three treatments (C = control, ME = *C. macellaria* egg, MI = *C. macellaria* first instar, MS = *C. macellaria* second instar, RE = *C. rufifacies* egg, RI = *C. rufifacies* first instar, RS = *C. rufifacies* second instar).

Conspecific microbial metabolic community profiles

Across generations

The MMCPs for the eggs and first instars of *C. macellaria* across all generations were significantly (df = 1, F = 3.62, *P* = 0.021) different. An ISA listed N-Acetyl-D-Glucosamine, Methyl-D-Glucoside, Phenylethylamine, and Hydroxy-Butyric Acid as the top four indicators of MMCP differences of the eggs and first instars of *C. macellaria*. There were no significant (*P* > 0.05) differences between MMCPs of the eggs and first instars across generations for *C. rufifacies*.

Generation one

There were no significant ($P > 0.05$) differences between the MMCPs for conspecific eggs and first instars in generation one.

Generation two

The MMCPs for *C. rufifacies* eggs and first instars were significantly (df = 1, F = 18.27, $P = 0.001$) different with no carbon indicators for MMCP differences. The introduction of second instars to NMDS analyses displayed significant (df = 2, F = 13.28, $P = 0.001$) differences between the eggs, first and second instars of *C. rufifacies* with an ISA listing D-Galcturonic Acid, Phenylethylamine, D-Cellobiose, and Cyclodextrin as carbon indicators of MMCP differences. There were no significant ($P > 0.05$) differences between the MMCPs for the eggs and first instars of *C. macellaria*. The introduction of second instars to NMDS analyses showed significant (df = 2, F = 3.35, $P = 0.027$) differences between *C. macellaria* eggs, first and second instars with an ISA listing Putrescine, Erythritol, D-Malic Acid, and Phenylethylamine as carbon indicators of MMCP differences.

CHAPTER IV

CONCLUSIONS

Microbial community functional shifts on immature blow fly stages

Heterospecifics

Microbial community function shifted and differentiated across fly species as *C. macellaria* and *C. rufifacies* progressed from egg to 2nd instar. Microbial community shifts across life stages are common in biology. Pechal et al. (2014) discovered microbial community functional shifts on swine carrion that mediated natural progression of arthropod succession to a short-lived ephemeral resource by the fluctuating presence of four main bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes), each having characteristic functional profiles and metabolic activities in the environment. While not functional, Singh et al. (2014) found microbial structural differentiation of the competing blow flies *Lucilia cuprina* (Wiedemann, 1830) and *Lucilia sericata* (Meigen, 1826) as each blow fly progressed from a larval to adult stage such as larvae having an approximate 2% relative abundance of Entercoccus species while the adult blow flies held an approximate 12% relative abundance of Entercoccus species during 454 sequencing.

Heterospecifics may be shifting their microbial communities during life stage progression in order to avoid interspecific competition. For example, Burkepile et al. (2006) indicated fresh carrion in a coastal marine ecosystem attracted 2.6 as many animals per bait trap as microbe-laden carrion, thus exhibiting how the bacteria present on carrion may alter their excretions to emit toxic chemicals that deter competing microorganisms and invertebrates. Additionally,

species of the fungus *Aspergillus* have exhibited shifts in metabolic activities to produce deterrents against dipteran competitors that promote larval mortality, thereby mediating the coexistence of the interspecific competitors (Trienens et al. 2010).

Conspecifics

For one generation, the MMCPs of all immature stages of both conspecifics were distinguishable. Alike heterospecifics, microbial shifts occur in conspecifics to avoid competition; however intraspecific microbial shifts may also occur as resource preferences change during life stage progression and as an accommodation to a change in physiology. Lam et al. (2007) indicated that ovipositing females of the house fly *Musca domestica* (Linnaeus, 1758) (Diptera: Muscidae) respond to bacterial cues associated with conspecific eggs. They determined that a threshold existed in which flies were repelled when volatiles were above the threshold and were attracted when below the threshold. They also determined the ecological relevance of this response. When forces to oviposit when volatiles were above the threshold, resulting eggs were cannibalized by other house fly larvae resulting from the older eggs releasing the higher concentration of volatiles.

Applications

Competition ecology

Competition ecology examines the intra- and interspecific interactions of organisms and how the presence of one organism affects the birth and death rates of another (Tilman 1994). I was able to differentiate two competing blow flies based on their MMCPs to better understand how the predator *C. rufifacies* is attracted to a resource that is colonized by *C. macellaria*. An example of

blow fly utilization of microbes to mediate competition and arrival of competitors to a resource is given in the study by Barnes et al (2010), which indicated that the bacterial communities present in the excretions and secretions of *L. sericata* and *Calliphora vicina* (Robineau-Desvoidy, 1830) had unique antimicrobial responses that may play a role in arthropod arrival to a resource (carrion). While not an insect example, Bever (2003) indicated soil microbial communities play an integral in the competition and coexistence of strong plant competitors, therefore an improved understanding of functional shifts of bacterial communities on any given organism may provide insight on how microorganisms play a role in mediating competition and coexistence on other competing organisms.

Forensic entomology

Forensic entomology is the use of arthropods as evidence in a court of law (Keh 1985). There is a distinct faunal succession associated with carrion that encompasses a wide variety of arthropods, many of which are necrophagous, sarcosaprophagous or predatory in nature (Greenburg 1991). Because decedents are usually discovered within the first few days of death, forensic investigators will typically encounter early stages of decomposition (Greenburg 1991). With *C. macellaria* and *C. rufifacies* being primary colonizers, immature stages (i.e., eggs, 1st instars, 2nd instars) are often discovered and collected as evidence to determine minimum postmortem intervals (mPMI) (Tomberlin et al. 2011b). During the egg and 1st instar stages, *C. macellaria* and *C. rufifacies* are indistinguishable in the field, therefore costly and extensive molecular analyses must be used for identification (Amendt et al. 2004). Because this study has demonstrated that immature stages of heterospecific blow flies can be distinguished by their

MMCPs, methods to determine MMCPs have the potential to cut the time and costs that come with identifying immature blow fly stages.

Limitations and future research

The presence of a generation effect is one potential limitation to this study. Because there were drastically different results for both conspecifics and heterospecifics between generation one and generation two, the determination of which generation to draw conclusions from is subjective. A better understanding of how MMCPs of heterospecifics and conspecifics change from generation to generation would require additional generation tests to distinguish a definitive pattern of microbial metabolic shifts. Additionally, the statistical power of this study could be improved by the addition of more samples per species and life stage. The Biolog Ecoplate™ also contains known limitations that could have an effect of the outcome of this study: maintaining the inoculum density for each carbon source, restricted culturability, and the ability to extract micoorganisms from an environmental sample (Preston-Mafham 2002; Stefanowicz 2006).

This study has demonstrated significant differences in microbial metabolic community profiles of the immature stages of *C. macellaria* and *C. rufifacies*, and can serve as a framework for future research on MMCPs associated with an organism. Future research should include validation studies in order to determine if this mechanism of differentiation is applicable in the field. Additionally, this study may be used as a template for those that wish to study varying populations of blow flies to determine the effect of heterogeneous genomes on the microbial functional profiles. Methods for determining MMCPs paired with metagenomic sequencing

have the potential to provide a better understanding of the roles microbes play in development, inter- and intraspecific interactions, and decomposition of ephemeral resources.

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